ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 2008, p. 1173–1175 Vol. 52, No. 3
0066-4804/08/$08.00+0 doi:10.1128/AAC.01212-07

Relationship between Antibiotic Use and Incidence of MexXY-OprM Overproducers among Clinical Isolates of *Pseudomonas aeruginosa*▽

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Received 13 September 2007/Returned for modification 11 November 2007/Accepted 23 December 2007

In a university hospital, time-series analysis revealed a significant relationship between antibiotic (aminoglycoside, fluoroquinolone, and cefepime) use and incidence of MexXY-OprM-overproducing *Pseudomonas aeruginosa*. In vitro experiments confirm that such mutants were readily selected from both PAO1 and clinical strains when grown in the presence of these antibiotics.

The MexXY-OprM pump is one of 10 multidrug efflux systems characterized so far in *Pseudomonas aeruginosa* (1). Its overproduction, which leads to low-level resistance to aminoglycosides, fluoroquinolones, and zwitterionic cephalosporins (cefepime, cefpirome), occurs following mutations in the gene encoding the regulator MexZ (*agrZ* mutants) or in another gene (*agrW* mutants) (8). Despite the high prevalence of clinical *P. aeruginosa* isolates overproducing MexXY-OprM (4, 7), in vivo emergence of this resistance mechanism has rarely been reported to date. The conditions that favor the emergence of such mutants in a hospital setting remain unclear. To address this issue, we (i) analyzed the temporal relationship between the prevalence of clinical isolates of *P. aeruginosa* displaying a typical efflux-based resistance to aminoglycosides and antibiotic use and (ii) performed in vitro selection experiments.

(This study was presented in part at the 17th European Congress of Clinical Microbiology and Infectious Diseases, Munich, Germany, 1 to 4 April 2007 [5].)

Incidence of clinical MexXY-OprM overproducers. Resistance patterns of 3,820 *P. aeruginosa* isolates (excluding cystic fibrosis isolates) from clinical specimens between 1999 and 2005 were determined by the Kirby-Bauer disk method on Mueller-Hinton agar (Bio-Rad, Marnes-La-Coquette, France), as recommended by the Antibiogram Committee of the French Society for Microbiology (CA-SFM; recommendation 2007, November 2007 [http://www.sfm.asso.fr]). A Sirscan automated image analyzer (I2A, Perols, France) was used to measure inhibition diameters and to compile resistance data. Duplicate isolates were defined on the basis of the patient identity and the antibiotic phenotype and deleted.

Since the determination of MexXY-OprM production, based on molecular analysis, is not suitable for large-scale studies (2) and the nonenzymatic resistance to aminoglycosides is mostly due to MexXY-OprM overproduction (4), we considered, as recommended by the CA-SFM, as potential MexXY-OprM efflux overproducers all clinical isolates (named clinical PA/XY isolates) that displayed reduced inhibition zones (<20 mm) around the tested aminoglycoside disks (amikacin, 30 μg; gentamicin, 15 μg; tobramycin, 10 μg). As a confirmation, 58 (73%) out of 80 randomly chosen clinical PA/XY isolates overexpressed *mexXY* when analyzed by reverse transcription-PCR (detailed below). On the other hand, we have previously showed that MexXY-OprM overproduction was rather infrequent (19 out of 85 isolates, 22%) in aminoglycoside-susceptible isolates (4).

Time-series analysis (TSA). We conducted a TSA of monthly incidence of clinical PA/XY isolates and of monthly consumption of various antibiotic classes. Eight classes of antibiotics representing more than 90% of antibiotics used in our hospital were tested (Table 1). Statistical tests were carried out as previously described (10). Briefly, autoregressive integrated moving-average models were used to analyze the temporal behavior of each variable as a function of its previous values, its trends, and any recent sudden changes. Once the basic characteristics of the series were established, dynamic time-series modeling techniques were used to evaluate relationships between antimicrobial use series, expressed in defined daily dose per 1,000 patient-days, and resistance series (incidence of PA/XY isolates per 1,000 patient-days). Specifically, polynomial distributed lag (PDL) models were used for the detection and quantification of lagged effects of antimicrobial use on incidence of PA/XY isolates. In a PDL model, the relationship between the independent variables (past incidence of PA/XY isolates and antimicrobial use) and the dependent variable (incidence of PA/XY isolates) should evolve smoothly over time, through the use of "polynomial lags." The optimum PDL model for the data sets was obtained by application of the "general-to-specific" econometric methodology. Using the approach proposed by Pankratz, we adjusted a linear transfer function model (11). The results showed a significant relationship between the aminoglycoside, the fluoroquinolone, and the antipseudomonal cephalosporin use series and the clinical PA/XY series (Table 1). Conversely, a negative correlation between nonantipseudomonal cephalosporin, penicillin, and carbapenem use series and clinical PA/XY series was found. Overall, this statistical analysis showed that 81.1% of the vari-

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▽ Published ahead of print on 7 January 2008.

TABLE 1. Transfer function model for incidence of *P. aeruginosa* displaying MexXY-OprM overproduction according to antibiotic use[a]

Antibiotic class[b] or term	Order(s)[c] (mo)	Size effect
Constant		3.885 ± 0.417
Aminoglycosides (AMK, TOB, GEN)	0, 3, 4, 6	0.142 ± 0.038
Antipseudomonal fluoroquinolones (CIP)	0, 6	0.048 ± 0.006
Fluoroquinolones poorly active on *P. aeruginosa* (NOR, OFX)	5	0.019 ± 0.004
Antipseudomonal cephalosporins (CAZ, FEP)	2	0.037 ± 0.007
Cephalosporins inactive on *P. aeruginosa* (CXM, FOX, CRO, CTX)	0, 2, 6	−0.083 ± 0.014
Penicillins inactive on *P. aeruginosa* (AMX ± CLA and CLX)	0, 1, 3	−0.015 ± 0.002
Antipseudomonal penicillins (PIP ± TZB, TIC ± CLA and ATM)	0, 3, 5	−0.037 ± 0.029
Carbapenems (IPM, ERT, MEM)	0, 2, 5, 6	−0.158 ± 0.046
AR[d]	3	0.840
MA[e]	1	−0.964

[a] CHU Jean Minjoz, Besançon, France, January 1999 to January 2005.
[b] AMK, amikacin; TOB, tobramycin; GEN, gentamicin; CIP, ciprofloxacin; NOR, norfloxacin; OFX, ofloxacin; CAZ, ceftazidime; FEP, cefepime; CXM, cefuroxime; FOX, cefoxitin; CRO, ceftriaxone; CTX, cefotaxime; AMX, amoxicillin; CLA, clavulanic acid; CLX, cloxacillin; PIP, piperacillin; TZB, tazobactam; TIC, ticarcillin; ATM, aztreonam; IPM, imipenem; ERT, ertapenem; MEM, meropenem; ±, with or without.
[c] Delay necessary to observe the effect (in months).
[d] AR, autoregressive term representing past values of resistance.
[e] MA, moving-average term representing disturbances or abrupt changes of resistance.

ations of the incidence of clinical PA/XY isolates were explained by the variations of antibiotic use.

In vitro mutant selection. The ability of the 33 antibiotics available in our hospital to select for PA/XY mutants at the MICs or at a concentration twice the MICs was determined in vitro using the reference strain PAO1 and seven genotypically independent susceptible clinical strains (Table 2) (4). PA/XY mutants were detected by replicating resistant clones on three different Mueller-Hinton plates containing gentamicin (5 μg/ml) or cefepime (5 μg/ml) or no antibiotic. Mutants that grow on both gentamicin and cefepime agar were considered to be potential PA/XY mutants. Five mutants per antibiotic and per strain were randomly chosen for determination of their drug resistance phenotype by agar diffusion. PA/XY mutants typically displayed low-level resistance to aminoglycosides, ciprofloxacin, and cefepime. The stability of their resistance phenotype was checked by serial subcultures during 10 days. The overexpression of *mexY* was assessed by reverse transcription-PCR (6), and the gene encoding the repressor MexZ was sequenced in one mutant per antibiotic and per strain. These in vitro experiments showed that PA/XY mutants were readily selected from both PAO1 and clinical strains when grown in the presence of fluoroquinolones, aminoglycosides, and cefepime (Table 2). All the selected mutants overexpressed *mexY* and displayed a low-level resistance to aminoglycosides, ciprofloxacin, and cefepime. Six out of 20 PA/XY mutants carried a mutated MexZ regulator (*agrZ* mutants; Table 2). As previously observed, no clear correlation between the type of mutants (*agrZ* or *agrW*), the nature of mutations in *mexZ*, the expression levels of *mexY*, and the resistance levels to effluxed antibiotics could be established (6, 8).

Such a concordance between the two independent approaches indicates that TSA is a powerful tool for investigating the relationship between antibiotic exposure and the occurrence of a particular resistance mechanism.

Our results indicate that, in the hospital setting, PA/XY mutants appear to be mostly selected by aminoglycosides, fluoroquinolones, and antipseudomonal cephalosporins. As ceftaz-

TABLE 2. In vitro selection of MexXY-OprM-overexpressing mutants from susceptible *P. aeruginosa* isolates

Strain(s) and selecting antibiotic (concn [μg/ml])[a]	Frequency of MexXY-OprM-overexpressing mutants	Mutant characteristics[b]					
		mexY expression (type of mutant)[c]	MIC (μg/ml) of:				
			GEN	TOB	AMK	CIP	FEP
PAO1		1.0	4	1	4	0.12	2
AMK (8)	8×10^{-7}	70.7 (*agrW*)	8–16	2–4	8–32	0.5	4–8
TOB (1)	2×10^{-7}	47.6 (*agrZ*)	8–16	2–4	8–32	0.5	4–8
GEN (4)	8×10^{-7}	51.6 (*agrZ*)	8–16	2–4	8–32	0.5	4–8
OFX (1)	7×10^{-7}	38.9 (*agrW*)	8–16	2–4	8–32	0.5	4–8
NOR (1)	1×10^{-6}	63.1 (*agrZ*)	8–16	2–4	8–32	0.5	4–8
CIP (0.25)	5×10^{-8}	18.3 (*agrW*)	8–16	2–4	8–32	0.5	4–8
FEP (4)	2×10^{-7}	36.4 (*agrW*)	8–16	2–4	8–32	0.5	4–8
Clinical isolates ($n = 7$)		0.5–3.1	2–4	1	4	0.06–0.25	1–2
AMK (8)	6×10^{-7}–1×10^{-7}	7.2–93.2	8–16	2–4	8–32	0.5	4–8
CIP (0.25)	9×10^{-7}–4×10^{-7}	13.8–343.3	8–16	2–4	8–32	0.5	4–8
FEP (4)	3×10^{-8}–8×10^{-7}	21.1–26.6	8–16	2–4	8–32	0.5	4–8

[a] The antibiotics that do not appear here do not select for PA/XY mutants (selection rates $< 10^{-11}$) (MICs are in parentheses): amoxicillin with or without clavulanic acid (32/4 μg/ml), aztreonam (4 μg/ml), azithromycin (4 μg/ml), cefotaxime (32 μg/ml), cefoxitin (4,096 μg/ml), ceftazidime (2 μg/ml), ceftriaxone (32 μg/ml), cefuroxime (256 μg/ml), chloramphenicol (64 μg/ml), cloxacillin (4,096 μg/ml), colistin (2 μg/ml), ertapenem (4 μg/ml), erythromycin (256 μg/ml), fosfomycin (256 μg/ml), imipenem (1 μg/ml), linezolid (2,048 μg/ml), meropenem (0,5 μg/ml), piperacillin (16 μg/ml), piperacillin plus tazobactam (4/0,5 μg/ml), rifampin (32 μg/ml), teicoplanin (1,024 μg/ml), ticarcillin with or without clavulanic acid (16/1,07 μg/ml), trimethoprim plus sulfamethoxazole (128 μg/ml), vancomycin (1,024 μg/ml). AMK, amikacin; TOB, tobramycin; GEN, gentamicin; CIP, ciprofloxacin; NOR, norfloxacin; OFX, ofloxacin; FEP, cefepime.
[b] One mutant per strain and per antibiotic was characterized. Substitutions (G<u>C</u>C→A<u>C</u>C, $A_{20}T$; TA<u>C</u>→TA<u>A</u>, Y_{204}stop; A<u>A</u>C→A<u>G</u>C, $N_{186}S$) or 1-bp deletions (A_{80} and C_{605} for two mutants) were observed in the *mexZ* gene.
[c] Expression relative to PAO1; mean values from two independent experiments.

idime does not select for PA/XY isolates in vitro, it seems likely that cefepime could select for PA/XY mutants in the hospital setting. This study provides good evidence that MexXY-OprM overproduction confers significant in vivo advantage to *P. aeruginosa* under selective pressure by aminoglycosides, fluoroquinolones, or cefepime. This finding is concordant with Monte Carlo simulations and clinical studies suggesting that low-level resistance conferred by efflux overproduction can substantially decrease the target attainment rates or clinical efficacy of fluoroquinolone, aminoglycoside, and cefepime treatments (3, 12). However, the breakpoints defined by North American or European experts (e.g., CLSI, the British Society for Antimicrobial Chemotherapy, CA-SFM, and the European Committee on Antimicrobial Susceptibility Testing) classify most PA/XY mutants as susceptible to these compounds. Moreover, increased drug efflux can be the first step toward higher resistance to fluoroquinolones (due to target mutation) in *P. aeruginosa* (9). Consequently, microbiologists should report this low-level resistance in order to optimize chemotherapy. Additional studies are needed to determine whether the emergence of PA/XY mutants is linked to insufficiently high doses of antibiotic and if it is due to the spread of clonal PA/XY strains rather than the trigger of MexXY-OprM overproduction in patients. Moreover, our findings together with other in vitro data (9) suggest that the use of an efflux inhibitor in combination with antipseudomonal antibiotics could be beneficial to prevent selection of first-step mutants prone to evolve toward pan-drug resistance.

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